Staphylococcus aureus Stimulates Neutrophil Targeting Chemokine Expression in Keratinocytes through an Autocrine IL-1α Signaling Loop

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Staphylococcus aureus is a significant human pathogen that can colonize the skin. Neutrophils are well known to be involved in clearance of the bacterium. This study focused on exploring the role that human keratinocytes have as first responders to bacterial challenges. IL-1α and IL-1β increased mRNA production and protein secretion of the neutrophil chemotactic CXCL1, CXCL2, and IL-8 in keratinocytes. *S. aureus* and the bacterial cell wall components lipoteichoic acid (LTA) and peptidoglycan (PGN) induced similar expression profiles in a Toll-like receptor (TLR)-2-dependent manner. Interestingly, the *S. aureus*-induced mRNA levels peaked at later time points than those induced by IL-1. The *S. aureus*-activated chemokine production was preceded by significant IL-1α and IL-1β secretion. Expression of IL-1α was significantly higher than that of IL-1β. Inhibition of IL-1RI using neutralizing antibodies revealed that *S. aureus*-derived LTA and PGN-induced chemokine expression requires IL-1RI engagement. Surprisingly, we further found that chemokine secretion is dependent upon endocrine IL-1α, but not IL-1β, signaling. Our data show that the innate immune response of keratinocytes is regulated differently than those of other cell types. This may represent a fail-safe system that protects the host against genetic variation and immune evasion mechanisms developed by pathogens.

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INTRODUCTION

The Gram-positive *Staphylococcus aureus* bacterium causes a wide spectrum of human inflammatory diseases, ranging from benign abscesses, through necrotizing fasciitis to potentially fatal pneumonia and sepsis. When challenged by a microorganism, the host must be able to immediately initiate elimination processes. These are in part activated by the innate immune system and the Toll-like receptors (TLRs). TLRs distinguish self from non-self by recognizing pathogen-associated molecular patterns present on, or in, the micro-organism but not the host (reviewed in Ishii *et al.*, 2008). Such motifs include flagellin from the flagellum of motile bacteria (recognized by TLR5), single- and double-stranded RNA generated during viral life cycles (sensed by TLR7/8 and TLR3, respectively), and lipopolysaccharide and lipoteichoic acid (LTA) from bacteria (bound by TLR4 and TLR2, respectively). TLRs are expressed not only on innate immune cells such as macrophages, neutrophils, and dendritic cells, but also on the adaptive B and T cells. Furthermore, the receptors can be found on fibroblasts and several types of epithelial cells (Akira *et al.*, 2006). The engagement of the TLRs activates complex intracellular signaling pathways that, through increased production of pro-inflammatory cytokines and type I IFNs, regulate downstream innate and adaptive immune responses (Akira *et al.*, 2006).

The *S. aureus* cell wall contains two potential TLR activators: LTA and peptidoglycan (PGN). LTA and PGN from many types of Gram-positive bacteria are typically recognized by TLR2 (Akira *et al.*, 2006) and several studies have independently implicated TLR2 in the immune response against *S. aureus*. TLR2-deficient mice have a significantly higher mortality rate than wild-type mice after intravenous administration of *S. aureus* (Takeuchi *et al.*, 2000), and production of tumor necrosis factor-α and IL-6 is lower in $TLR2^{-/-}$ than $TLR2^{+/+}$ macrophages treated with heat-killed *S. aureus* (HKSA) (Takeuchi *et al.*, 2000). TLR2-deficient mice are more readily colonized in the nasal cavity than wild-type mice (Gonzalez-Zorn *et al.*, 2005), and establishment of subcutaneous infections requires 10-fold more colony-forming units in $TLR2^{+/+}$ than in $TLR2^{-/-}$ mice (Kristian *et al.*, 2003). Curiously, neutrophil recruitment and clearance of subcutaneous bacteria seem to be TLR2 independent in mice (Miller *et al.*, 2006). These phenotypes are instead dependent upon IL-1β and IL-1R type I (IL-1RI) signaling (Miller *et al.*, 2006, 2007).

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Abbreviations: HKSA, heat-killed S. aureus*; KERTr, CCD 1106 KERTr cells; LTA, lipoteichoic acid; PAb, polyclonal antibody; PGN, peptidoglycan; TLR, Toll-like receptor*

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IL-1 is a pluri-potent pro-inflammatory cytokine (reviewed in O'Neill, 2008; Dinarello, 2009). There are two classical isoforms of IL-1, IL-1α and IL-1β. Although both proteins initially are expressed as intracellular proteins, they are released from cells in distinct manners. The IL-1α precursor is active and is released from dying cells (Dinarello, 2009). In contrast, the IL-1β precursor requires processing by the inflammasome and caspase-1 before it is secreted by cells in a purine receptor P2X7-dependent manner (Franchi *et al.*, 2009). IL-1α and IL-1β activate expression of many immunologically important genes through the transmembrane receptor complex, comprising IL-1RI and IL-1R accessory protein (IL-1RAcP), which is present on most, if not all, cell types (O'Neill, 2008). The intracellular domain of IL-1RI is homologous to the intracellular domains of the TLRs (O'Neill, 2008). As a result of this similarity, these distinct receptor classes activate largely the same intracellular signaling pathways and transcription factors, including mitogen-activated protein kinases, activator protein-1, and NF-κB (O'Neill, 2008). Consequently, such diverse phenotypes as fever, vasodilation, proliferation, differentiation, adhesion, and migration (Akira *et al.*, 2006; Dinarello, 2009) are synchronized to ensure an appropriate immune response.

It has been established that keratinocytes, which are the main constituents of the epidermal layer of the skin, express TLRs and produce cytokines when the cells are exposed to TLR ligands (Lebre *et al.*, 2006 and references therein). However, the specific involvement of keratinocytes in initiating and orchestrating immune responses to skin infections, including *S. aureus* colonization, is largely unknown. In this study we report how *S. aureus* stimulates production of neutrophil chemoattractive cytokines in human keratinocytes.

RESULTS

IL-1-stimulated keratinocytes express CXC chemokine mRNAs and proteins involved in neutrophil chemotaxis

Neutrophils have an essential role in eliminating *S. aureus* from the host (Verdrengh and Tarkowski, 1997; Mölne *et al.*, 2000; DeLeo *et al.*, 2009). It was recently shown that IL-1β, but not IL-1α, is required for recruitment of neutrophils to a subcutaneous *S. aureus* infection (Miller *et al.*, 2007). However, because of the experimental model, this study did not address the role of the epidermis and specifically the keratinocytes. Given the capability of *S. aureus* to colonize the skin and because many infections start as abrasions of the skin, we were interested in evaluating the role of human keratinocytes in recruiting neutrophils. Using microarray analyses of chemokine mRNA expression in response to IL-1, we have previously observed that mRNAs encoding the neutrophil targeting chemokines, such as CXCL1 and CXCL2, were upregulated by IL-1 (Sanmiguel *et al.*, 2009). However, these observations were not further examined.

To validate the CXCL1- and CXCL2-specific array data, primary human neonatal keratinocytes were treated with increasing concentrations of IL-1β. After 1.5, 3, 6, and 24 hours, production of mRNA and protein was determined using real-time reverse transcription-PCR (RT-PCR) and ELISA, respectively. Expression levels of CXCL1 and CXCL2 mRNA and protein were induced in time- and IL-1β concentration-dependent manners (Figure 1a and b). Interestingly, after only 1.5 hours, rapid bursts in CXCL1 and CXCL2 mRNA levels were observed in IL-1β-treated keratinocytes (Figure 1a). Subsequently, levels of the CXCL1 and CXCL2 mRNAs gradually declined. At the 1.5-hour time point, the CXCL1 and CXCL2 mRNA levels were approximately 30- and 15-fold higher (respectively, $P<0.01$) in 5 ng ml^{-1} IL-1β-treated keratinocytes than in medium-only-treated cells (Figure 1a).

Significantly increased secretion of CXCL1 ($P<0.05$) and CXCL2 ($P<0.01$) could be detected already at 1.5 hours after IL-1β treatment (Figure 1b). Despite declining mRNA levels after 1.5 hours, CXCL1 and CXCL2 protein concentrations continued to increase throughout the duration of the experiment (Figure 1b). The differences in mRNA and protein expression profiles likely reflect differential stabilities of these molecules. At the 24-hour time point, levels of CXCL1 and CXCL2 in medium from cells treated with 5 ng ml^{-1} IL-1β were approximately 25- and 5-fold, respectively, which was higher ($P<0.01$) than in medium from cells not receiving IL-1β.

It has previously been shown that keratinocyte production of IL-8 (also known as CXCL8) is induced by *S. aureus* (Mempel *et al.*, 2003; Sasaki *et al.*, 2003). Because of the proximity of the microarray targets for mRNAs encoding IL-8 and the highly expressed Toll-interacting protein, TOLLIP, our previous expression analyses could not evaluate the expression of IL-8 (Sanmiguel *et al.*, 2009). Expression of IL-8 mRNA and protein was examined in this study as described above. In contrast to the expression of the CXCL1 and CXCL2 mRNAs (Figure 1a), IL-8 mRNA levels peaked at 3–6 hours after stimulation. Approximately 25-fold increases ($P<0.01$) in IL-8 mRNA expression were observed in 5 ng ml^{-1} IL-1β-stimulated keratinocytes compared with untreated cells after 3 and 6 hours (Figure 1c). Levels of IL-8 protein in medium from cells treated with IL-1β increased in a time- and concentration-dependent manner throughout the experiment (Figure 1c). At the 24-hour time point, approximately 12-fold ($P<0.05$) higher levels of IL-8 were observed in medium from cells treated with 5 ng ml^{-1} IL-1β than cells treated with medium only (Figure 1c).

We have previously shown that IL-1α and IL-1β activate gene expression in the same manner in keratinocytes (Sanmiguel *et al.*, 2009). In this study IL-1α and IL-1β also induced similar CXCL1, CXCL2, and IL-8 mRNA and protein expression profiles (data not shown). IL-1-induced expression of CXCL1, CXCL2, and IL-8 was confirmed using several independent batches of neonatal and adult primary keratinocytes and the stable cell lines, HEK001 and CCD 1106 KERTr cells (KERTr; data not shown).

CXC chemokine expression is regulated by *S. aureus* and cell wall components derived from the bacteria

Bacteria are recognized by the host TLRs (Ishii *et al.*, 2008) that activate an intracellular signaling cascade shared by IL-1RI (Akira *et al.*, 2006; Boraschi and Tagliabue, 2006;

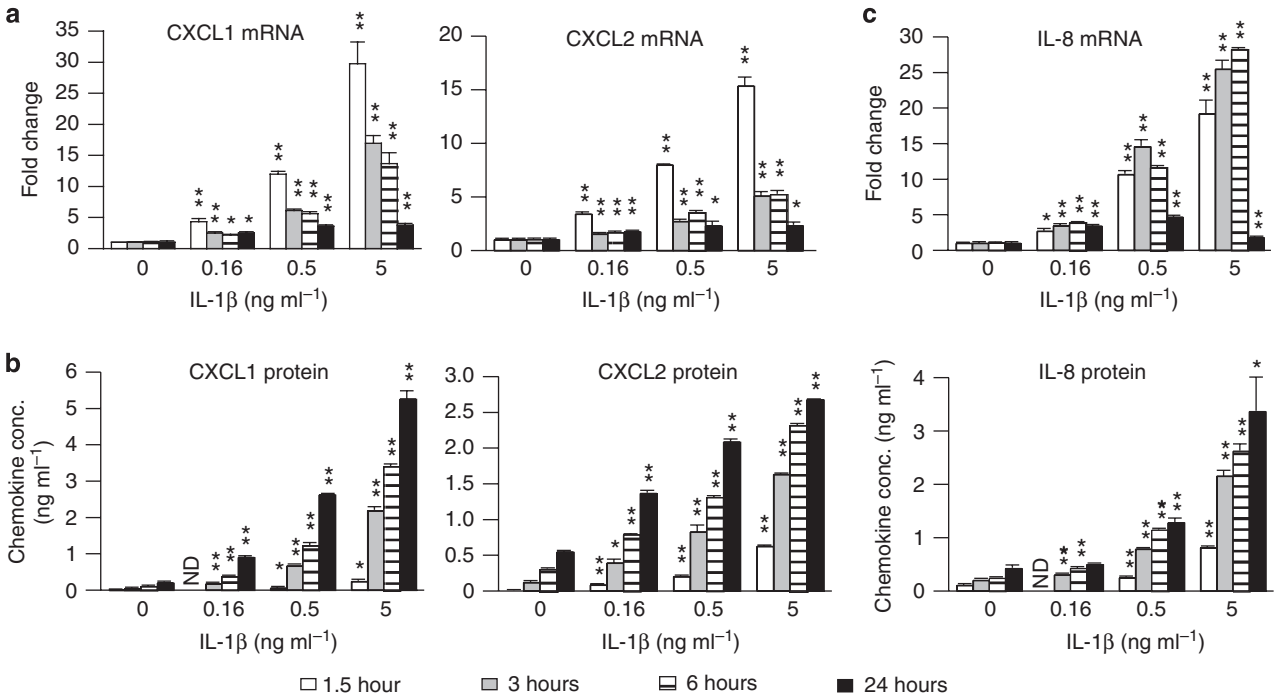

Figure 1. IL-1 induces CXC chemokine expression in keratinocytes. Primary neonatal keratinocytes (lot 143) were treated with medium only or increasing concentrations of IL-1β as indicated. Total RNA and culture medium were collected after 1.5, 3, 6, and 24 hours. (**a**) CXCL1 and CXCL2 mRNA levels were determined using real-time reverse transcription PCR (RT-PCR) and the comparative C_T method. CXCL1 and CXCL2 mRNA levels were normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels and graphically represented as mean fold change ± SD in comparison to cells treated with medium only. (**b**) CXCL1 and CXCL2 protein levels (mean ± SD) in the culture medium were determined using ELISA. (**c**) IL-8 mRNA and protein levels were determined as described in (**a**) and (**b**). *$P<0.05$ (compared with cells treated with medium only and collected at the same time point). **$P<0.01$ (compared with cells treated with medium only and collected at the same time point). ND, not determined.

O'Neill, 2008). We therefore wondered whether *S. aureus* would activate expression of the same CXC chemokines as IL-1 (Figure 1). It is known that keratinocytes express antimicrobial peptides, including the anti-staphylococcal β-defensin 3, which is upregulated by *S. aureus* (Menzies and Kenoyer, 2006). To ensure a consistent degree of keratinocyte activation throughout our experiments, we chose to use heat-killed bacteria. Primary keratinocytes were treated with increasing amounts of HKSA, and expression of CXCL1, CXCL2, and IL-8 was examined as described above after 1, 3, 6, and 24 hours (Figure 2). Time- and concentration-dependent increases in all three CXC chemokine mRNA levels were observed after HKSA stimulation (Figure 2a). Curiously, HKSA-induced mRNA levels peaked later (Figure 2a) than in response to IL-1 (Figure 1a and c). The CXCL1 mRNA levels reached maximum induction (2- to 5-fold, $P<0.05$) after 3 hours with all three amounts of HKSA (Figure 2a). Levels of the CXCL2 and IL-8 mRNAs peaked (9- and 32-fold induction, respectively, $P<0.01$) at 6 hours with the highest concentration of HKSA (Figure 2a).

In line with the observed shift in maximum CXC chemokine mRNA induction by HKSA compared with IL-1, significantly increased protein expression was also slightly delayed (Figure 2b compared with Figure 1b and c). The highest levels of CXCL1, CXCL2, and IL-8 induced by HKSA after 24 hours (Figure 2b) were comparable to those produced in response to IL-1 (Figure 1).

LTA and PGN from *S. aureus* are known to activate gene expression in macrophages (Takeuchi *et al.*, 1999; Hashimoto *et al.*, 2006). To evaluate whether the LTA and PGN derived from *S. aureus* activate CXC chemokine expression in keratinocytes in a manner similar to that induced by the bacterium, cells were in addition to HKSA (Figure 2) treated with LTA and PGN in increasing amounts (see Supplementary Figure S1 online). RNA and protein expression was evaluated after 1, 3, 6, and 24 hours. Both LTA and PGN led to increased CXCL1, CXCL2, and IL-8 (Supplementary Figure S1a–c online) mRNA expression. The mRNA levels peaked at 3–6 hours (Supplementary Figure S1a–c online) in agreement with that observed for HKSA (Figure 2a). Secretion of CXCL1, CXCL2, and IL-8 (Supplementary Figure S1d–f online) protein was also induced by both LTA and PGN. Although LTA activated gene expression at lower concentrations than PGN, PGN gave rise to a higher degree of induction than LTA (Supplementary Figure S1 online). Similar observations were made using several independent batches of neonatal and adult primary keratinocytes, and the stable cell lines HEK001 and KERTr (data not shown). These observations show that *S. aureus* can activate neutrophil targeting CXC chemokine production in keratino-

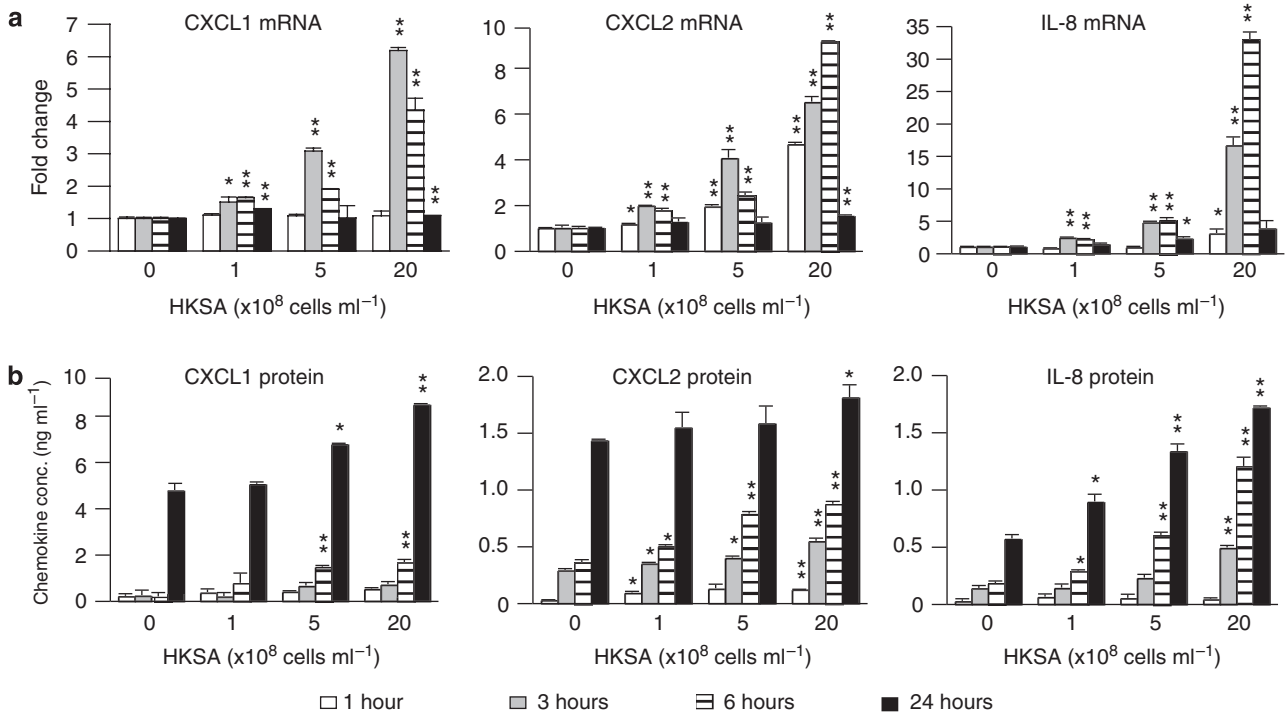

Figure 2. *Staphylococcus aureus* increases CXC chemokine expression in keratinocytes. Primary neonatal keratinocytes (lot 164) were treated with medium only or increasing amounts of HKSA as indicated. Total RNA and culture medium were collected after 1, 3, 6, and 24 hours. (**a**) CXCL1, CXCL2, and IL-8 mRNA levels were determined using real-time reverse transcription-PCR (RT-PCR) and the comparative C_T method. CXCL1, CXCL2, and IL-8 mRNA levels were normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels and graphically represented as mean fold change ± SD in comparison to cells treated with medium only. (**b**) CXCL1, CXCL2, and IL-8 protein levels in the culture medium were determined using ELISA. *$P<0.05$ (compared with cells treated with medium only and collected at the same time point). **$P<0.01$ (compared with cells treated with medium only and collected at the same time point).

cytes, possibly through the bacterial cell wall components LTA and PGN.

S. aureus-, LTA-, and PGN-driven CXC chemokine expression is TLR2 dependent

It has previously been shown that *S. aureus* activates expression of antimicrobial peptides (Menzies and Kenoyer, 2006) and IL-8 (Mempel *et al.*, 2003; Sasaki *et al.*, 2003) in human keratinocytes through TLR2 (Mempel *et al.*, 2003; Menzies and Kenoyer, 2006). Curiously, it has also been reported that neutrophil recruitment to subcutaneous *S. aureus* infection sites in mice was independent of TLR2 (Miller *et al.*, 2006).

To test whether TLR2 is involved in driving CXC chemokine expression in keratinocytes exposed to *S. aureus* and its subcomponents, KERTr cells were incubated with MAb human TLR2-specific antibody (Figure 3a–c). Control cells received isotype-matched mouse IgG_{2a}. After 2 hours, medium, HKSA, LTA, or PGN were added to the medium and cells were incubated for an additional 20 hours, after which CXC chemokine secretion into the medium was examined. A borderline significant ($P=0.07$) 32% decrease in HKSA-induced CXCL1 expression (Figure 3a) was observed in samples from MAb hTLR2-incubated cells compared with control cells. CXCL1 production in response to LTA and PGN was significantly ($P<0.01$ and $P<0.05$, respectively) reduced by approximately 50% in the presence of MAb hTLR2 compared with control IgG_{2a} (Figure 3a). HKSA-, LTA-, and PGN-induced production of CXCL2 (Figure 3a) and IL-8 (Figure 3a) by cells incubated with MAb hTLR2 was all also significantly ($P<0.01$ or $P<0.05$) reduced by 30–50% compared with cells incubated with mouse IgG_{2a}.

To verify the involvement of TLR2, the above experiments were repeated using a polyclonal (PAb) antibody against hTLR2 (Figure 3d–f). Although increased expression ($P<0.01$) of CXCL1 (Figure 3d), CXCL2 (Figure 3e), and IL-8 (Figure 3f) was observed in response to HKSA, LTA, and PGN in the presence of normal rat IgG, there was no induction of these chemokines in the presence of the PAb hTLR2 (Figure 3d–f).

Lower concentrations of the antibodies (MAb and PAb and appropriate control IgGs) led to less dramatic, but significant, inhibition of CXC chemokine expression (data not shown). Similar data were obtained using primary keratinocytes and HEK001 cells (data not shown). These observations show that TLR2 regulates CXC chemokine expression in keratinocytes exposed to *S. aureus* and cellular components thereof.

IL-1α and IL-1β mRNA and protein levels increase in response to *S. aureus*, LTA, and PGN

Our previous microarray analyses of inflammatory gene expression in keratinocytes identified *IL1A* and *IL1B* as putative target genes regulated by IL-1 (Sanmiguel *et al.*, 2009). In analogy with the similar induction of CXC chemokines by IL-1 (Figure 1) and *S. aureus* (Figure 2 and

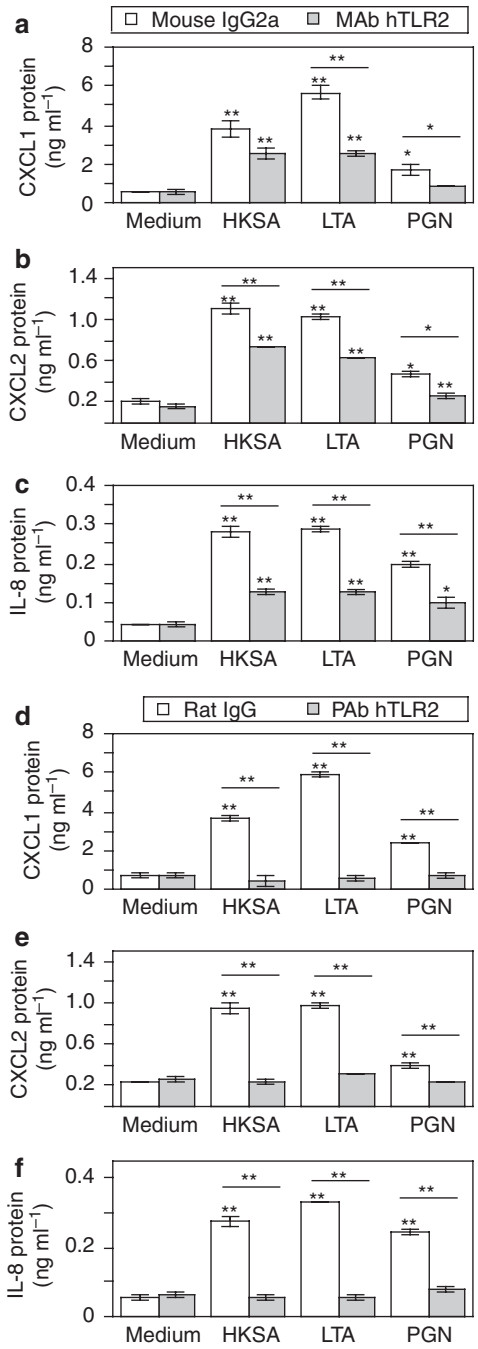

Figure 3. CXC chemokine expression is TLR2 dependent. KERTr cells were pre-treated with TLR2-specific antibodies (gray bars; MAb, monoclonal mouse (**a–c**) or PAb, polyclonal rat (**d–f**)). Control cells were treated with species- and isotype-matched normal IgG (open bars; mouse IgG_{2a} (**a–c**) or rat IgG (**d–f**)). After 2 hours, medium, HKSA, LTA, or PGN was added to the medium. Secretion of the CXC chemokines, CXCL1 (**a, d**), CXCL2 (**b, e**), and IL-8 (**c, f**), was determined after 20 hours by ELISA. Data are shown as mean ± SD. *$P<0.05$ (compared with same Ig- and medium-treated cells unless marked otherwise with bar below). **$P<0.01$ (compared with same Ig- and medium-treated cells unless marked otherwise with bar below).

Supplementary Figure S1 online), we speculated that IL-1α and IL-1β production could be regulated in response to HKSA, LTA, and/or PGN. To test this hypothesis, expression of IL-1α and IL-1β mRNA and protein was examined (Figure 4

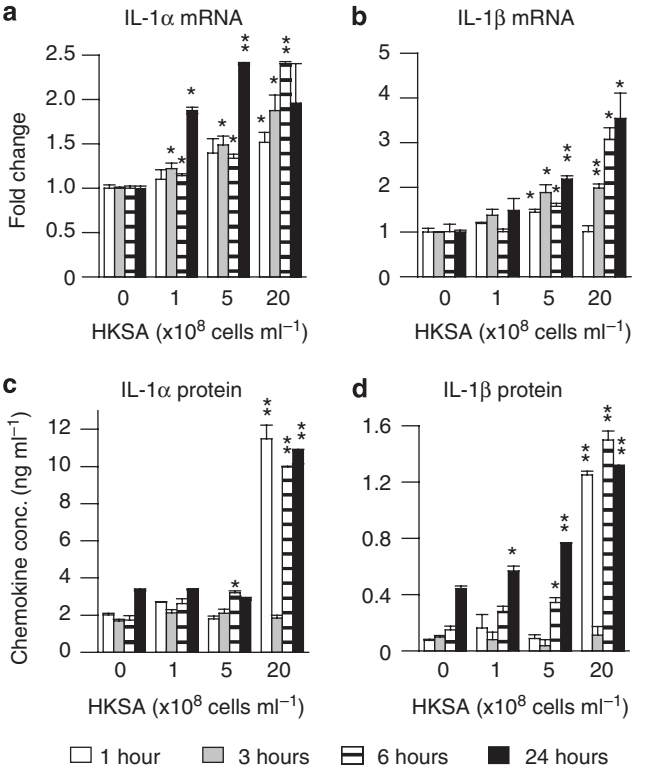

Figure 4. IL-1α and IL-1β expression and secretion are enhanced by *Staphylococcus aureus*. Primary neonatal keratinocytes (lot 164) were treated with medium only or increasing amounts of heat-killed *S. aureus* (HKSA) as indicated. Total RNA and culture medium were collected after 1, 3, 6, and 24 hours. (**a**) IL-1α and (**b**) IL-1β mRNA levels were determined using real-time reverse transcription-PCR (RT-PCR) and the comparative C_T method. IL-1α and IL-1β mRNA levels were normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels and graphically represented as mean fold change ± SD in comparison to cells treated with medium only. (**c**) IL-1α and (**d**) IL-1β protein levels (mean ± SD) in the culture medium were determined using ELISA. *$P<0.05$ (compared with cells treated with medium only and collected at the same time point). **$P<0.01$ (compared with cells treated with medium only and collected at the same time point).

and Supplementary Figure S2 online) as described above. Only a modest 1.5-fold increase in IL-1α ($P<0.05$, Figure 4a) and no change in IL-1β (Figure 4b) mRNA levels were observed in cells treated with the highest amount of HKSA after 1 hour. Surprisingly, dramatic increases in IL-1 secretion were observed after only 1 hour (Figure 4c and d). An approximately 6-fold increase in IL-1α ($P<0.01$, Figure 4c) and a 15-fold increase in IL-1β ($P<0.01$, Figure 4d) protein levels were observed at the 1-hour time point in medium from cells treated with HKSA. Similar effects were observed with LTA and PGN (Supplementary Figure S2 online). Given the lack of a comparable mRNA induction at the same early time point, these observations strongly suggest that pre-formed and intracellular stored pools of IL-1α and IL-1β protein are released from keratinocytes immediately after exposure to *S. aureus*.

Significantly increased (1.5- to 3.5-fold, $P<0.05$) IL-1α and IL-1β mRNA levels were observed at the 3, 6, and/or 24 h

time points after HKSA, LTA, and PGN treatment (Figure 4 and Supplementary Figure S2 online) compared with medium-only treated cells. These increases in mRNA levels correlated with 1.5- to 3.5-fold elevated protein levels ($P<0.05$, Figure 4 and Supplementary Figure S2 online), although a transient decrease (compared with the 1-hour time point) in protein levels was observed at the 3-hour time point (Figure 4c and d).

Within individual samples, approximately 10-fold more IL-1α than IL-1β was detected (Figure 4c and d). On the basis of the RT-PCR C_T values, IL-1α mRNA levels were approximately 8 times higher than IL-1β mRNA levels in untreated cells (data not shown).

In summary, IL-1α is expressed at higher levels than IL-1β at both the mRNA and protein level in keratinocytes. After exposure to *S. aureus*, or its subcomponents, keratinocytes rapidly, within 1 hour, release IL-1α and IL-1β into the culture medium. Subsequently, IL-1α and IL-1β mRNA levels are increased and *de novo* synthesized protein is secreted.

TLR ligand-induced CXC chemokine expression is driven by IL-1RI signaling

The observed CXC chemokine mRNA expression profiles induced by IL-1 (Figure 1) and *S. aureus* (Figures 2 and 3) differed with respect to the time at which the mRNA levels peaked (before 3 hours for IL-1 and between 3 and 6 hours for HKSA, LTA, and PGN). Furthermore, increased IL-1 secretion was present within an hour of HKSA, LTA, or PGN stimulation (Figure 4 and Supplementary Figure S2 online). These observations led us to hypothesize that IL-1 secretion and subsequent activation of IL-1RI on the keratinocytes contribute to the *S. aureus*-induced chemokine expression.

The involvement of IL-1RI was examined by incubating primary adult keratinocytes with polyclonal antibodies specific for human IL-1RI (PAb hIL-1RI) before treatment with medium only, LTA, or PGN (Figure 5a–c). Control cells were incubated with normal goat IgG. Efficacy of the antibody was verified by evaluating inhibition of IL-1α- and IL-1β-induced production of CXCL1, CXCL2, and IL-8 (Figure 5d).

Pre-incubation of cells with PAb hIL-1RI led to an approximately 50% reduction ($P<0.01$) in constitutive expression of CXCL1 (Figure 5a), CXCL2 (Figure 5b), and IL-8 (Figure 5c) compared with cells receiving the control IgG. Cells treated with PAb hIL-1RI completely failed to respond to PGN with increased production of CXCL1 and CXCL2 (Figure 5a and b). PGN-induced production of IL-8 in these cells amounted to only approximately 80 pg ml^{-1} above levels generated by PAb hIL-1RI and medium-only-treated cells. In comparison, normal goat IgG and PGN-treated cells produced approximately 250 pg ml^{-1} more IL-8 than normal goat IgG and medium-only-treated cells (Figure 5c). Although PAb hIL-1RI-treated cells did respond to LTA, the levels of increased production were significantly lower than those induced in appropriately matched control cells (Figure 5a–c). For example, normal goat IgG and LTA-treated cells secreted approximately 5 ng ml^{-1} more CXCL1 than cells treated with normal goat IgG and medium only (Figure 5a). In comparison, only approximately 0.9 ng ml^{-1} more CXCL1 was released from PAb hIL-1RI and LTA-treated cells than the PAb hIL-1RI and medium-only reference cells (Figure 5a).

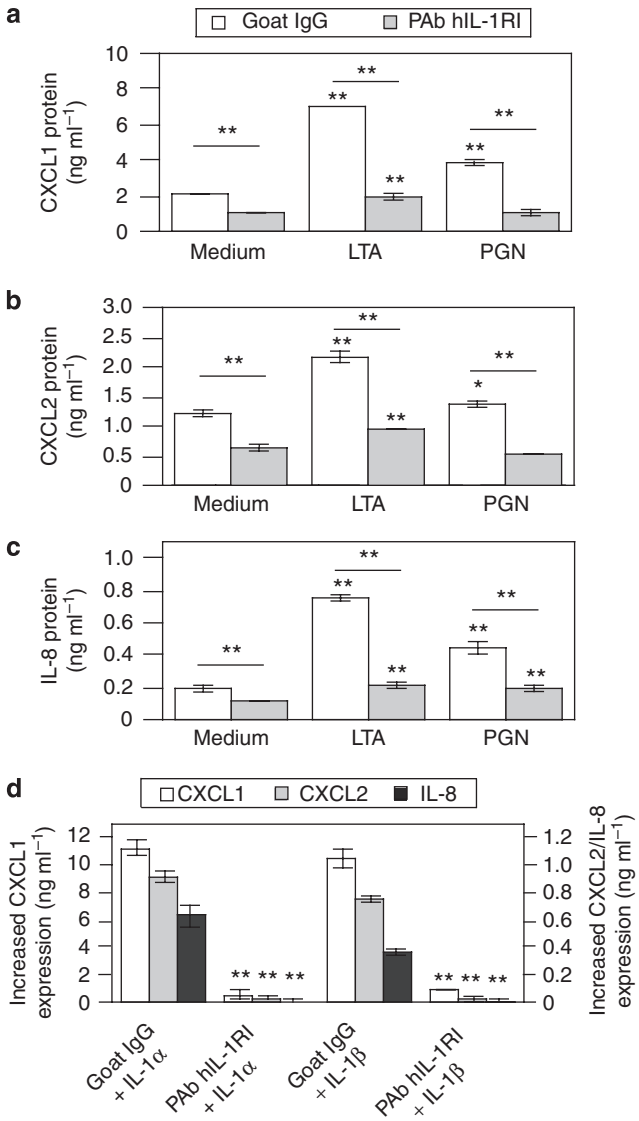

Figure 5. *Staphylococcus aureus*-induced CXC chemokine expression is dependent upon IL-1RI signaling. (**a–c**) Primary adult keratinocytes (lot 070504–901) were pre-treated with goat anti-human IL-1RI antibodies (polyclonal antibody (PAb) hIL-1RI, gray bars) or normal goat IgG (open bars). After 2 hours, medium, lipoteichoic acid (LTA), or peptidoglycan (PGN) was added to the medium. Secretion of the CXC chemokines (**a**) CXCL1, (**b**) CXCL2, and (**c**) IL-8 was determined after 20 hours by ELISA. Data are shown as mean ± SD. *$P<0.05$ (compared with same Ig- and medium-treated cells unless marked otherwise with bar below). **$P<0.01$ (compared with same Ig- and medium-treated cells unless marked otherwise with bar below). (**d**) Cells were pre-treated with PAb hIL-1RI or normal goat IgG for 2 hours. Medium, 10 ng ml^{-1} IL-1α, or 10 ng ml^{-1} IL-1β was added to the culture medium, and expression of CXCL1 (open bars), CXCL2 (light gray), and IL-8 (dark gray bars) was examined after 20 hours. Increased chemokine expression in samples from IL-1-treated cells was calculated by subtracting protein levels in Ig-matched samples from cells not receiving IL-1. Data are shown as mean ± SD. **$P<0.01$ (compared with isotype-matched normal IgG and same IL-1 isoform).

Similar observations were made using HEK001 and KERTr cells (data not shown). The above data show that LTA- and PGN-mediated CXC chemokine expression requires IL-1RI engagement.

Constitutive IL-1α expression contributes to constitutive CXC chemokine expression

To analyze further the mechanism by which IL-1RI mediates *S. aureus*-induced CXC chemokine production, we explored whether the IL-1 secreted by the keratinocytes contributed to the gene activation. As we observed that inhibition of IL-1RI signaling reduced constitutive production of the CXC chemokines (Figure 5a–c), we initially examined the effect of IL-1α and IL-1β on constitutive CXCL1, CXCL2, and IL-8 expression. Cells were treated with polyclonal antibodies specific for human IL-1α (PAb hIL-1α) or a monoclonal antibody recognizing human IL-1β (MAb hIL-1β). Control cells were incubated with appropriate species- and isotype-matched normal IgG. Although neutralization of IL-1α reduced chemokine expression by approximately 50% ($P<0.01$), the IL-1β-specific antibody did not affect chemokine expression (Figure 6a).

To confirm the activities and specificities of the antibodies, cells were pre-treated with PAb hIL-1α, MAb hIL-1β, or appropriate control IgGs. After 2 hours, cells were further treated with medium only, IL-1α, or IL-1β, and chemokine expression was determined after an additional 20-hour incubation. As expected, PAb hIL-1α blocked IL-1α-induced chemokine expression ($P<0.01$) but had no effect upon IL-1β signaling (Figure 6b). The MAb hIL-1β was confirmed to efficiently ($P<0.01$) neutralize 10 ng ml^{-1} IL-1β (Figure 6b), a concentration significantly exceeding that produced by the keratinocytes (Figure 4d).

One may speculate that the apparent effect of the PAb hIL-1α could be because of a nonspecific recognition of an unrelated protein. To confirm the role of IL-1α in regulating constitutive production of CXC chemokines, cells were treated with a monoclonal mouse anti-human IL-1α antibody (MAb hIL-1α) or normal mouse IgG_{2a} before analyses of CXCL1, CXCL2, and IL-8 expression (Figure 6c). In agreement with the observations made with the polyclonal antibody, chemokine expression by MAb hIL-1α-treated cells were approximately 30% ($P<0.01$) of that observed with mouse IgG_{2A}-treated cells (Figure 6c). Similar data were obtained using the stable cell lines HEK001 and KERTr (data not shown). The above observations establish that IL-1α, but not IL-1β, regulate constitutive CXC chemokine secretion by keratinocytes.

Variation in constitutive production of the CXC chemokines was observed between different batches of both neonatal (Figures 1 and 2) and adult primary keratinocytes (Figures 6 and 7) and the stable cell lines KERTr (Figure 3) and

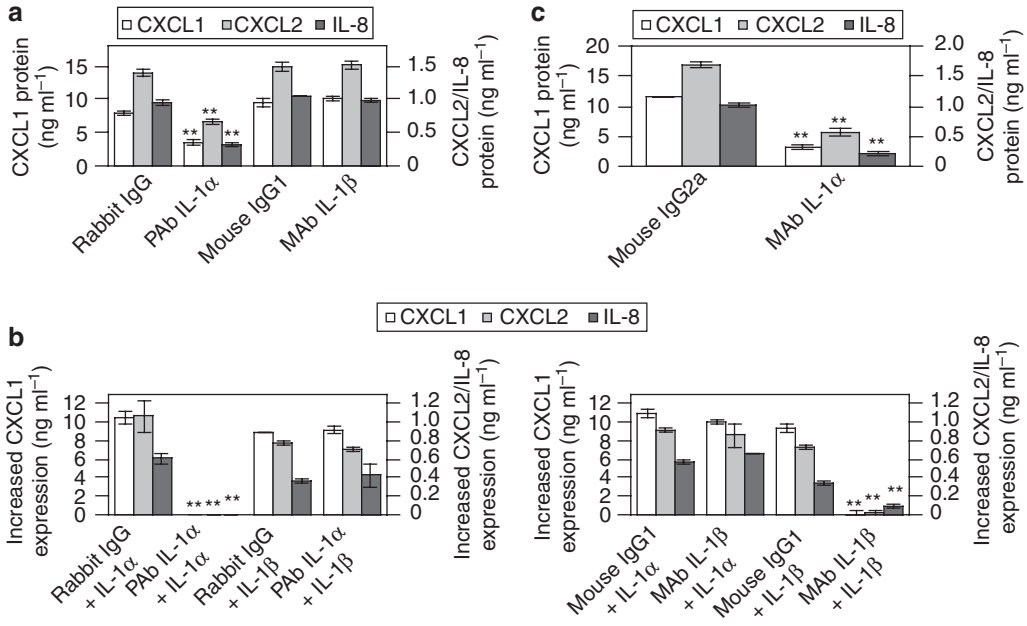

Figure 6. IL-1α, but not IL-1β, contributes to constitutive CXC chemokine expression. (**a, c**), Primary adult keratinocytes (lot 452309) were treated with polyclonal rabbit anti-human IL-1α (polyclonal antibody (PAb) hIL-1α, **a**), monoclonal mouse anti-human IL-1β (MAb hIL-1β, **a**), or monoclonal mouse anti-human IL-1α antibodies (MAb hIL-1α, **c**). Control cells were treated with species- and isotype-matched normal IgG. Secretion of the CXC chemokines CXCL1 (open bars), CXCL2 (light gray bars), and IL-8 (dark gray) was determined after 22 hours by ELISA. Data are shown as mean ± SD. $^{**}P<0.01$ (compared with isotype-matched normal IgG). (**b**) Cells were pre-treated with PAb hIL-1α, MAb hIL-1β, or appropriate species- and isotype-matched normal IgG for 2 hours. Medium, 10 ng ml^{-1} IL-1α, or 10 ng ml^{-1} IL-1β was added to the culture medium, and expression of CXCL1 (open bars), CXCL2 (light gray), and IL-8 (dark gray bars) was examined after 20 hours. Increased chemokine expression in samples from IL-1-treated cells was calculated by subtracting protein levels in Ig-matched samples from cells not receiving IL-1. Data are shown as mean ± SD. $^{**}P<0.01$ (compared with isotype-matched normal IgG and same IL-1 isoform).

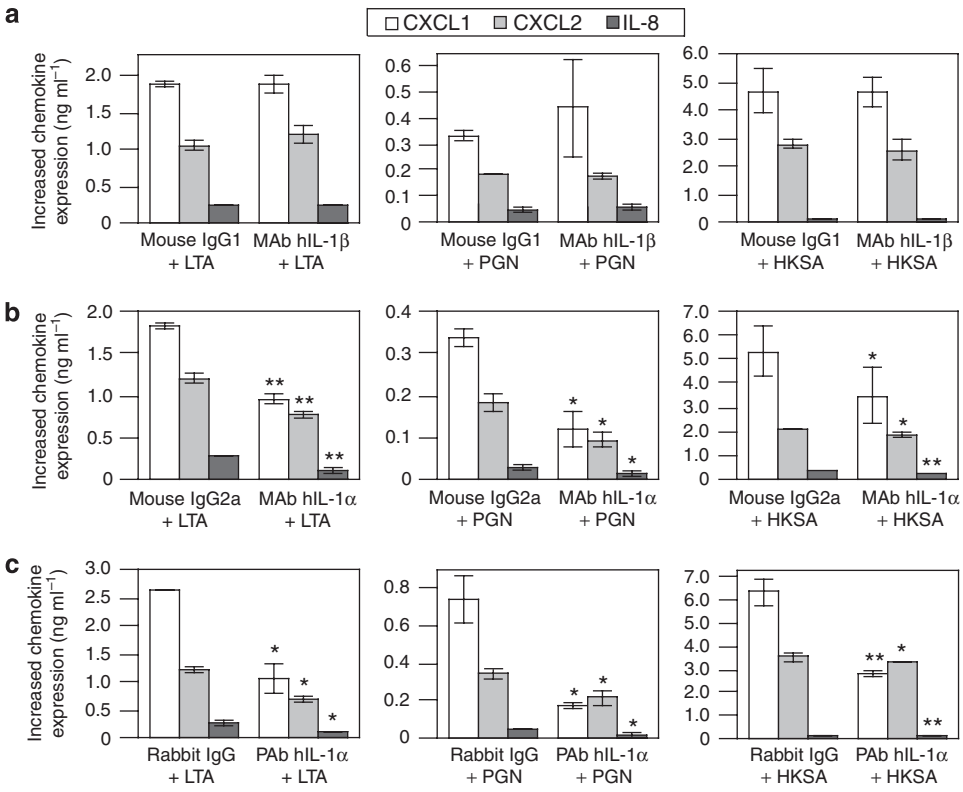

Figure 7. ***Staphylococcus aureus*-induced CXC chemokine expression is IL-1α, but not IL-1β, dependent.** Primary adult and neonatal keratinocytes (lot 452309 and lot 158, respectively) were pre-treated with (**a**) MAb hIL-1β, (**b**) MAb hIL-1α, or (**c**) polyclonal antibody (PAb) hIL-1α. Control cells were incubated with appropriate species- and isotype-matched normal IgG. After 2 hours, medium, LTA, PGN (adult cells), or HKSA (neonatal cells) was added to the culture medium, and expression of CXCL1 (open bars), CXCL2 (light gray), and IL-8 (dark gray bars) was examined after 20 hours. Increased chemokine expression in samples from LTA-, PGN-, or HKSA-treated cells was calculated by subtracting protein levels in Ig-matched samples from cells not receiving LTA, PGN, or HKSA. Data are shown as mean ± SD. *$P<0.05$ (compared with isotype-matched normal IgG). **$P<0.01$ (compared with isotype-matched normal IgG).

HEK001 (data not shown). These lot-to-lot disparities likely reflect gene polymorphisms in *CXCL1, CXCL2,* or *IL8,* and/or genes regulating expression of these; e.g., *IL1A*.

Induced CXC chemokine expression is dependent upon IL-1α but not IL-1β

Studies of subcutaneous *S. aureus* infections in mice have indicated that IL-1RI and IL-1β, but not IL-1α, are required for neutrophil recruitment (Miller *et al.*, 2006, 2007). As keratinocytes secrete elevated levels of IL-1 in response to HKSA, LTA, and PGN (Figure 4 and Supplementary Figure S2 online), we wondered whether this could be an autocrine mechanism regulating increased production of the neutrophil targeting chemokines such as CXCL1, CXCL2, and IL-8. We initially performed neutralization experiments as described above testing the involvement of IL-1β. Surprisingly, we found that the functionally active (Figure 6b) MAb hIL-1β did not affect chemokine induction (Figure 7a). In contrast, both antibodies directed against IL-1α significantly ($P<0.05$) inhibited HKSA-, LTA-, and PGN-activated CXCL1, CXCL2, and IL-8 expression (Figure 7b and c). The levels of increased production of the three chemokines in the presence of the IL-1α-neutralizing antibodies were decreased to 25–50% ($P<0.05$) of that observed with control cells treated with normal IgG (Figure 7b and c). Similar observations were made using HEK001 cells (data not shown). Our data show that both constitutive- and *S. aureus*-induced neutrophil targeting CXC chemokine expression in keratinocytes is regulated by an endocrine signaling pathway involving IL-1α, but not IL-1β.

DISCUSSION

It is well established that neutrophils have an important role in eliminating *S. aureus* from the host in general (Verdrengh and Tarkowski, 1997; DeLeo *et al.*, 2009 and references therein) and from the skin specifically (Mölne *et al.*, 2000). *S. aureus* infections may initially involve colonization of the skin and/or the nasal cavity and compromised epithelial barrier function (van Belkum, 2006). When the skin is injured the keratinocytes are likely to be the first to encounter an invading potential pathogen. To ensure that the challenge is cleared before the microorganism can multiply and spread, the keratinocytes must send danger signals into the surrounding tissue to summon, for example, neutrophils. The data reported in this study show that *S. aureus*-exposed keratinocytes produce chemokines (CXCL1, CXCL2, and IL-8; Figures 2 and 3) that are chemotactic to neutrophils. Interestingly, the TLR2-dependent (Figure 3) chemokine induction requires an endocrine IL-1α, but not IL-1β, signaling loop (Figures 4–7). Initially, it may be puzzling that although the keratinocytes

produce both IL-1α and IL-1β, only IL-1α has a functional effect. However, it should be noticed that the keratinocytes secrete significantly ($P<0.01$) more IL-1α (Figure 4c) than IL-1β (Figure 4d) and the levels of IL-1β synthesis may be insufficient to significantly affect gene expression.

It has previously been reported that IL-1 signaling, through IL-1RI, is required for neutrophil recruitment to a subcutaneous *S. aureus* challenge in mice (Miller *et al.*, 2006). It was furthermore showed that IL-1β, but not IL-1α, was the functional isoform (Miller *et al.*, 2007). We chose to examine specifically the role that keratinocytes have as first responders to microbial challenges. Our data show that chemokine expression in keratinocytes is regulated differently than in the (un-indentified) cell types involved in the *in vivo* model; i.e., it is TLR2 and IL-1α, but not IL-1β, dependent. The different outcomes (IL-1α vs. IL-1β dependence) of using various models may reflect repetition, yet variation, in the immune system that is required to ensure proper protection against immune evasion mechanisms developed by pathogenic microorganisms and genetic variation in the host. *S. aureus* has acquired several virulence factors aimed at inhibiting neutrophil chemotaxis (reviewed in Rooijakkers *et al.*, 2005; Kraus and Peschel, 2008). A multi-layered IL-1 system involving keratinocytes and IL-1α (as reported in this study) as initial responders in the skin and IL-1β as the activator used by other cell types in the epidermis and underlying tissues (Miller *et al.*, 2007) may provide a fail-safe scheme in which one mechanism can substitute for the other should this fall short of its task. It should be noted that we did observe expression of IL-1β in keratinocytes after activation by *S. aureus* (Figure 4). The levels of IL-1β expressed by the keratinocytes seemed to be too low to have a functional effect in our model (Figures 6 and 7). However, there may be situations in which IL-1α is inactive, e.g., mutations in the host, or becomes depleted. In these situations IL-1β may substitute for IL-1α in the epidermis.

The review literature on TLR and IL-1RI signaling often mentions that these receptors activate the same (Akira *et al.*, 2006; Boraschi and Tagliabue, 2006) or similar signaling pathways (O'Neill, 2008). Not surprisingly, we observed that the TLR2 ligands (HKSA, LTA, and PGN;, Figure 2 and Supplementary Figure S1 online) increased expression of the same CXC chemokine genes activated by IL-1 (Figure 1). Given that the used TLR2 ligands also led to secretion of IL-1 (Figure 4), we anticipated that IL-1 would be partially responsible for the TLR2 ligand-induced CXC chemokine expression. Surprisingly, we found that TLR2 ligand-induced CXC chemokine expression could be completely blocked by antibodies directed against IL-1RI (Figure 5). This implies that TLR2 signaling does not directly regulate CXC chemokine expression, but requires IL-1 secretion and signaling as an intermediate step (TLR2→IL-1→IL-1RI→CXC chemokine). This may further indicate that the IL-1RI and TLRs do not strictly activate the same signaling pathways. The intracellular regions of IL-1RI and the TLRs contain a conserved protein–protein interaction domain involved in binding the adapter protein MyD88 (Akira *et al.*, 2006; O'Neill, 2008; Dinarello, 2009). One known difference between IL-1RI and TLR2 signaling is the specific requirement for the adapter protein toll-interleukin 1 receptor domain containing adaptor protein (TIRAP) in TLR2, but not IL-1RI, signaling. TIRAP facilitates the recruitment of MyD88 to the TLR2 receptor complex (Akira *et al.*, 2006; O'Neill, 2008). It has been shown that knockout of TIRAP impairs TLR2 (and TLR4)-mediated cytokine expression in macrophages and dendritic cells. Induction of the same cytokines by other TLR ligands or IL-1 was not affected (Horng *et al.*, 2002; Yamamoto *et al.*, 2002). It is possible that TIRAP, through an unknown mechanism, could facilitate a differential gene expression profile compared with those induced by MyD88-restricted pathways. An alternative, or perhaps complementary, hypothesis is that scaffolds have an essential role in regulating which genes are activated in response to certain stimuli. Such scaffolds could involve the Pellino proteins as previously described (Jensen and Whitehead, 2003a, b), or some of the many proteins involved in regulating mitogen-activated protein kinase signaling (reviewed in Morrison and Davis, 2003).

TLR2 requires a coreceptor, TLR1 or TLR6, for ligand binding; however, the specific functional involvement of these cofactors remains controversial (Fournier and Philpott, 2005). We observed that hTLR2 MAb had only a borderline significant effect upon CXCL1 expression (Figure 3a), whereas the hTLR2 PAb abolished CXCL1 expression (Figure 3d). It is likely that the used mono- and polyclonal antibodies have differential effects upon heterodimer formation between TLR2 and the cofactors TLR1 and TLR6. Furthermore, the cofactors may have diverging roles in downstream signaling. Further studies are required to determine whether TLR1 and TLR6 have specific functions in terms of the range of chemokines expressed in response to distinct *S. aureus*-derived ligands.

Interestingly, we observed variations in the constitutive levels of the three neutrophil chemotactic chemokines, CXCL1, CXCL2, and IL-8 (compare Figures 1, 2, 5 and 6; medium-only-treated samples), between lot numbers of primary cells. This could be due to genetic variations in the CXC chemokine genes and/or genes regulating their expression. Differences in constitutive expression of chemokines, and other innate immune factors, may determine whether an individual becomes an intermittent or chronic carrier of *S. aureus*. Furthermore, as the starting degree of expression may influence the levels during inflammation, polymorphisms in the CXC chemokine genes, or their regulators, may also affect the outcome of an infection. Comprehensive studies involving genome-wide analyses of gene expression and polymorphisms may reveal patterns associated with *S. aureus* carrier status and infections. Analyses of such profiles in the context of further characterization of the multifaceted immune system activated in response to *S. aureus* may reveal mechanisms by which effective prophylactic and/or treatment strategies can be achieved.

MATERIALS AND METHODS

Cell cultures

Neonatal foreskin (pooled) and adult torso human primary keratinocytes were obtained from Invitrogen (Carlsbad, CA). The human

keratinocyte cell lines HEK001 (CRL-2404) and CCD 1106 KERTr (KERTr, CRL-2309) were obtained from ATCC (Manassas, VA). Cells were maintained and treated in defined keratinocyte serum-free medium supplemented with 50 μg ml^{-1} gentamicin (Invitrogen).

Activation of cell cultures. Cell cultures were grown to approximately 80–90% confluence and treated with medium only, IL-1α (National Cancer Institute, Frederick, MD), IL-1β (PeproTech, Rocky Hill, NJ), HKSA (Invivogen, San Diego, CA), purified LTA (Invivogen), and PGN (Invivogen) as indicated in figures. The HKSA, LTA, and PGN were all derived from *S. aureus* strain ATCC 6538. Bacteria were purchased heat-killed from Invivogen. In brief, bacteria were killed by one or more 15-minute cycles of autoclaving at 121 °C. Cells were pelleted by centrifugation and resuspended in water. Lack of viability was confirmed by solid- and liquid-state cultures for 5 days.

Neutralization experiments. Mouse monoclonal IgG_{2a} (MAb hTLR2) and polyclonal rat (PAb hTLR2) antibodies directed against human TLR2 were obtained from Invivogen. Polyclonal rabbit antibody against human IL-1α (500-P21A) was from PeproTech. Monoclonal mouse antibodies specific for human IL-1α (IgG_{2a}, MAB200) and IL-1β (IgG_1, MAB601), and polyclonal goat antibodies against human IL-1RI (AF269) were obtained from R&D Systems (Minneapolis, MN). Purified normal goat IgG, mouse IgG_1, mouse IgG_{2A}, rabbit IgG, and rat IgG were obtained from R&D Systems. For inhibition of TLR2 signaling, cells were pre-incubated with 50 μg ml^{-1} MAb hTLR2, 100 μg ml^{-1} PAb hTLR2, normal mouse IgG_{2a}, or normal rat IgG for 2 hours. In IL-1 signaling neutralization experiments, cells were pre-incubated with 10 μg ml^{-1} antigen-specific antibodies or species- and isotype-matched control antibodies for 2 hours. After pre-incubating medium only, HKSA (10^9 cells ml^{-1} final concentration), LTA (10 μg ml^{-1} final concentration), or PGN (250 μg ml^{-1} final concentration) was added to the antibody containing medium, and incubation was continued for an additional 20 hours before chemokine expression was determined by ELISA. All experiments were performed at least three times with similar outcomes.

RNA isolation and real-time RT-PCR

Total RNA was extracted using the RNeasy purification system according to the manufacturer's instructions (Qiagen, Valencia, CA). Reverse transcription (RT) and real-time PCR was performed as described elsewhere (Sanmiguel *et al.*, 2009). In brief, 1 μg total RNA was reverse transcribed using Avian Myeloblastosis Virus reverse transcriptase (Promega, Madison, WI) and oligo$(dN)_6$ primer (GE Healthcare, Piscataway, NJ). Real-time RT-PCR was performed using RT^2 Real-Time SYBR Green PCR Master Mix (SABiosciences, Frederick, MD) on an Opticon2 instrument (Bio-Rad, Hercules, CA). Primer pairs (Supplementary Table S1 online) specific for individual mRNA/complementary DNAs were designed such that PCR products (80–100 bp) span exon—exon junctions, thereby preventing amplification of genomic DNA. Assays were validated using serial dilutions and confirmation of equal amplification efficiencies of the complementary DNA of interest and the glyceraldehyde-3-phosphate dehydrogenase complementary DNA. Fold differences in expression were calculated using the comparative C_T method by standardizing against glyceraldehyde-3-phosphate dehydrogenase expression and comparing expression in cytokine- or TLR ligand-treated cells to expression in cells treated with medium only.

ELISA

CXCL1 and IL-1β concentrations in culture medium from stimulated cells were determined using the appropriate DuoSet ELISA development kits (R&D Systems). CXCL2, IL-1α, and IL-8 levels were determined using ELISA development assays from PeproTech.

Statistical analyses

Data are shown as mean values and SD from one representative experiment of at least three independent experiments. Data were analyzed using the Student's *t*-test when appropriate.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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